Original Article

Can Subconjunctival Bevacizumab Injection Regress Corneal Neovascularization?

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Purpose: To determine the effect of subconjunctival bevacizumab injection on corneal neovascularization.

Material and Methods: The study included 16 eyes of 8 New Zelland albin rabbits. A 6/0 silk suture was placed intrastromally near the superior limbus in bot eyes of rabbits to induce corneal neovascularization. Eyes were checked weekly fc neovascularization and adjacent conjunctival injection. Subconjunctival injections c 1.25mg and 2.5mg bevacizumab were given in right and left eyes respectivel Eyes were examined under the operating microscope. Digital photographs wer taken weekly for 6 weeks and then evaluated by using a computerized imag processing technique and a transparent grid technique to quantify the area c corneal neovascularization and adjacent conjunctival injection.

Results: Corneal neovascularization and adjacent conjunctival injection wer observed by the end of first week. Injections were performed at the second weel Only four rabbits could complete the study. Conjunctival injection improve significantly at the first week following injection. Most significant reduction c corneal neovascularization was observed in the second week in both eyes of all rabbits. No recurrence was noted although no repeat injection was given.

Conclusion: Subconjunctival injection of bevacizumab may become an adjunct in the treatment of corneal neovascularization.

Neovascularization is the formation of new vascular structures in areas that were previously avascular[1]. Although cornea is a transparent and a vascular tissue, it may be subject to neovascularization due to prolonged inflammation which eventually can lead to corneal opacification, impaired vision and induce corneal graft rejection after corneal transplantation.[1-3]

Vascular endothelial growth factor (VEGF) is a potent stimulator of endothelial cell growth in vitro and neovascularization in vivo.[4] The role of VEGF was well-established in different clinical conditions such as tumors, proliferative diabetic retinopathy, age-related macular degeneration[5-7] and various anti-VEGF agents have been utilized for neovascular eye diseases.

VEGF was an important endogenous factor for wound and inflammation-related corneal neovascularization in the rat model as well[8]. Bevacizumab is a recombinant humanized monoclonal antibody developed against VEGF which binds to soluble VEGF and prevents receptor binding thus inhibits endothelial cell proliferation and vessel formation.[9]

This animal study was conducted in order to assess whether subconjunctival bevacizumab injection could regress corneal neovascularization.

MATERIAL AND METHODS

Sixteen eyes of 8 New Zelland Albino rabbits (2 – 3 kilograms) were included. The study was approved by the Institutional Animal Care and Use Ethics Committee of Dokuz Eylul University, School of Medicine. All procedures were performed in accordance with the ARVO statement for the use of

Animals in Ophthalmic and Vision Research. All animals were housed in individual cages and maintained under standard conditions.

The cornea and anterior segment were examined in both eyes under the operating microscope to confirm normal anatomy and absence of previous neovascularization prior to the study.

Corneal Neovascularization Model

Rabbits were anesthetized with intramuscular ketamine hydrochloride (35mg/kg) and xylazine (5mg/kg) in the animal laboratory. For the topical anesthesia, one drop of proparacaine hydrochloride 0.5% (Alcaine®, Alcon) was instilled bilaterally. A double pass 6/0 silk suture was placed intrastromally near the superior limbus in order to induce neovascularization. All eyes were observed weekly for the development of corneal neovascularization.

Drug Administration

After the neovascularization was observed, bevacizumab (Altuzan®, Roche) was injected subconjunctivally by the help of a 27 G needle adjacent to the neovascularized area. Right eyes received 1.25mg (0.05ml) and left eyes received 2.5mg (0.1ml) of bevacizumab.

Assessment of Neovascularization

Digital photographs were taken weekly by Nikon Coolpix 4500, 4.0 megapixel camera, with 4X optic zoom under constant magnification and distance. All photographs were then evaluated by a computerized image processing technique to visualize the change in neovascularization and also by using a transparent grid pattern in order to quantify and compare the regression in neovascularization.

Computerized Image Processing

At the beginning of the process, a region of interest (ROI) was selected on the photographs taken before and after subconjunctival injection. This ROI was converted to grayscale, the contrast was enhanced for better rendering of vascularization and two images were registered. (Image registration is the process of aligning two or more images of the same scene. Typically, one image is considered the reference to which the second image is compared). The object of image registration is to bring the reference image into alignment with the second image by applying a spatial transformation. This spatial transformation maps locations in reference image to new locations in the second one. The spatial transformation used for registration is the 'local weighted mean' method which is applied by selecting 12 control points from each image and processing this points by using Matlab 7.04 Image Processing Toolbox.

After registering the two images, another ROI was selected where the vascularization was dense (Fig.1a and 1b). Then an edge detection procedure was applied to detect the vasculature in both images (Fig. 2a and 2b). This procedure looks for places in the image where the intensity changes rapidly, using one of these two criteria: 1) Places where the first derivative of the intensity is larger in magnitude than a threshold. 2) Places where the second derivative of the intensity has a zero crossing. Finally, the resulting images were automatically thresholded by using the method described by Otsu.[10] We got the vasculature in two binary images. The subtraction of these two images resulted with a final image where the difference between two vasculature was visible (Fig. 3).

Quantification of regression by point counting

The technique described by Howie et al[11] was used. A transparent grid pattern composed of uniform, equidistant points (4mm apart) was placed over each photograph and the number of points coinciding with the area of neovascularization was counted. The numbers representing the area of neovascularization just before bevacizumab injection were considered as baseline and the baseline number of points was compared to the number obtained from the photos taken at each week.

Statistical analysis

Mann-Whitney U test was used to compare the numbers obtained from transparent grid technique in order to assess the efficacy of 1.25mg and 2.5mg of bevacizumab injections on neovascularization. Wilcox on analysis was performed in order to evaluate the regression in neovascularization at each week.

RESULTS

Corneal neovascularization developed in all eyes by the end of first week after placing the stromal suture (Fig. 4). Circumferential and radial corneal neovascularization was observed originating from the area of adjacent limbal injection at the second week. After the

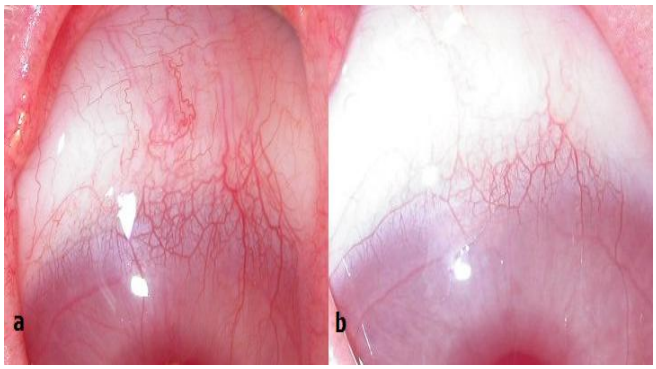

Fig. 1: **1a** **2b**

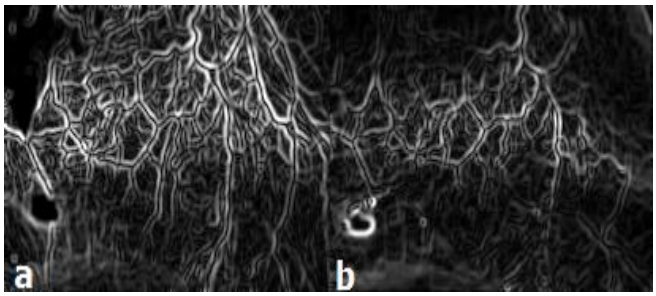

Fig. 2: **a** **2b**

Fig. 3:

injection of bevacizumab (Fig. 5), some decrease in baseline corneal neovascularization was noted within the first week, this change was not significant. The regression of conjunctival injection was most significant at the first week when compared to the following weeks. The most significant reduction of corneal neovascularization, was observed between the baseline evaluation and the second week (Fig. 6) in both eyes of all 4 rabbits whereas the difference between the second week and the following weeks was not significant. The graph in Fig. 7 shows the

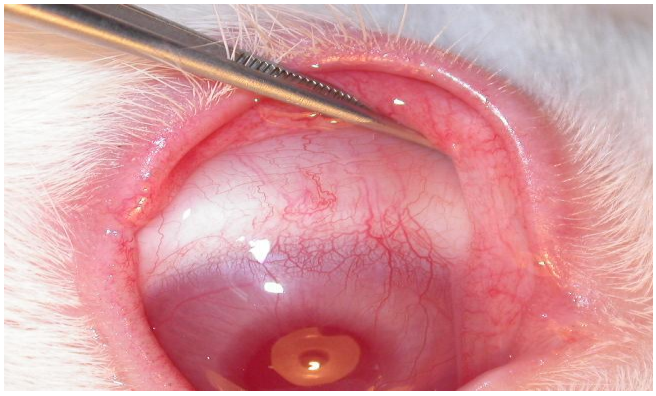

Fig. 4:

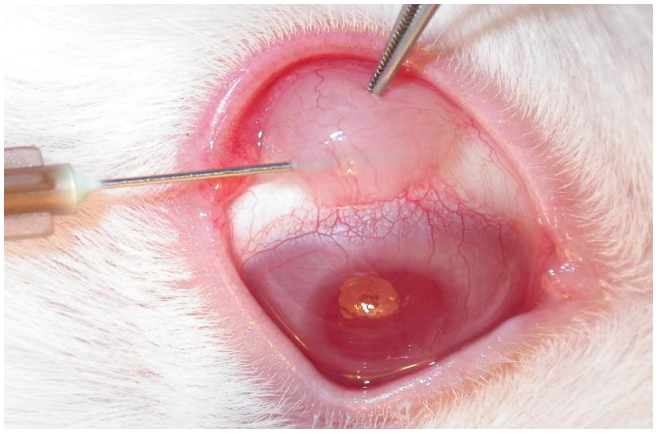

Fig. 5:

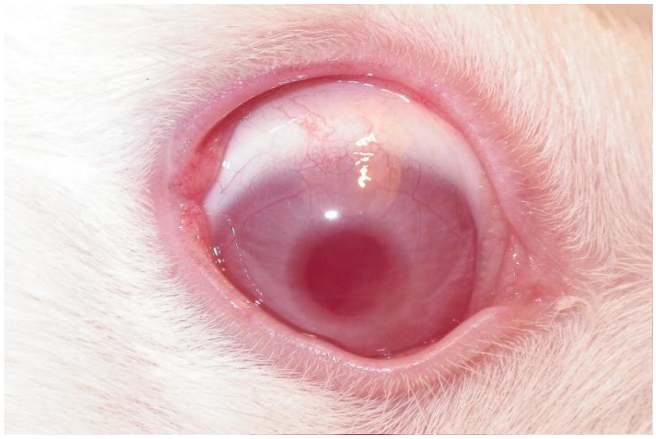

Fig. 6:

regression in conjunctival injection and corneal neovascularization during the study period. No difference in regression of neovascularization was observed between right and left eyes, which received 1.25mg and 2.5mg of bevacizumab respectively. Regression persisted and no complications were encountered during six weeks of follow-up. Although

no repeat doses of subconjunctival bevacizumab were given, neovascularization did not recur Fig. 8.

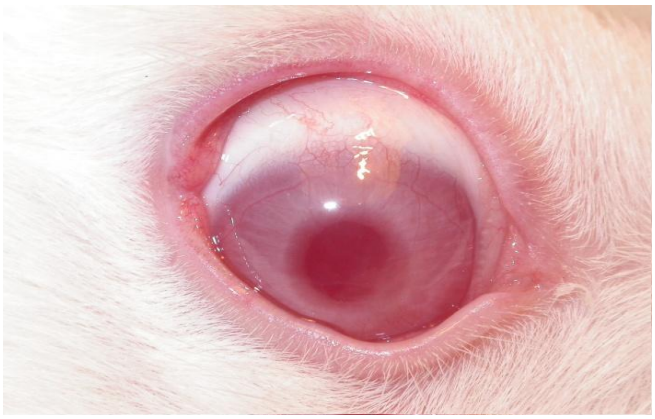

Fig. 7:

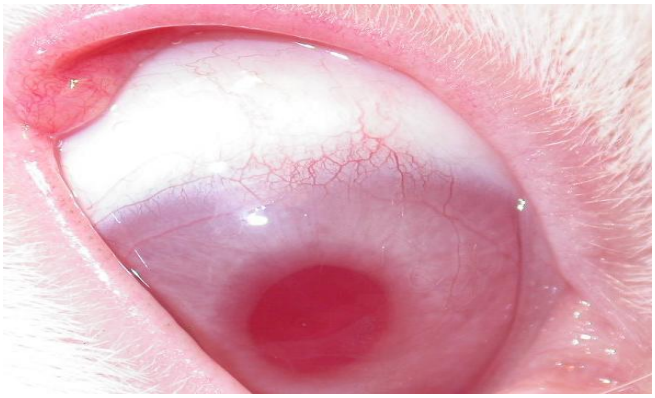

Fig. 8:

DISCUSSION

The pathogenesis of corneal angiogenesis has not yet been clearly defined.[12] However, it is obvious that prevention of corneal neovascularization would help maintain the transparency and immune privilege of the cornea. The role of VEGF in inflammatory corneal neovascularization has been investigated recently. VEGF was up regulated in inflamed and vascularized corneas in human and in animal models.[8, 12]

Amano and co-workers[8] used a rat model in order to quantify corneal VEGF mRNA levels with ribonuclease protection assay. They removed corneal and limbal epithelium in order to induce circumferential corneal neovascularization and identified a positive correlation between corneal VEGF mRNA levels and neovascularization. Constitutive VEGF mRNA was very low in normal cornea while it was expressed greater than 10-fold higher levels after wounding. The majority of VEGF immunoreactivity was localized in the invading inflammatory cells. The specific inhibition of VEGF bioactivity with implantation of pellets containing controlled–release polyclonal anti-VEGF antibody into corneal stroma potently suppressed corneal neovascularization.[8]

Another rat study of established corneal transplant model for rejection evaluated VEGF production in the graft by immunohistochemistry.[13] Twenty-one rats underwent corneal transplantation. Grafted rats were divided into three groups each receiving 0.9 M NaCl, rabbit serum immunoglobin, or anti-VEGF antibody topically. From the day of operation, the treatment was applied five times daily for 10 days on the left eyes. Immunohistochemical analysis showed that immune deposits for VEGF existed in infiltrative cells in grafts and corneal epithelial cells. Histologic sections showed a moderate to marked mononuclear cell infiltration in saline and anti-rabbit IgG-treated grafts. With anti-VEGF antibody–treated grafts, mononuclear cell infiltration was mild compared with saline and anti-rabbit IgG-treated grafts. Neovascularization and edema were significantly suppressed from day 6 to day 19 in anti-VEGF antibody-treated grafts compared with rabbit IgG and saline-treated grafts.

Joussen et al[14] showed that VEGF had a regulatory effect in the conjunctivalization of the corneal surface. They reported that corneal neovascularization preceded the appearance of the goblet cells and demonstrated that VEGF is required for both corneal neovascularization and appearence of goblet cells after extensive limbal injury.

Manzano et al[15] administered bevacizumab topically on experimental corneal neovascularization in rats. After chemical cauterization of the rat corneas, daily instillation of bevacizumab 4mg/ml drops inhibited the corneal neovascularization significantly.

In this study, we have investigated the effect of subconjunctival bevacizumab injection on corneal neovascularization. We have observed significant reducetion of corneal neovascularization with subconjunctival injections of 1.25mg and 2.5mg of bevacizumab without any significant difference between the two doses. The regression was most prominent at the second week after injection when compared to baseline or other weeks. Interestingly, significant decrease in the adjacent conjunctival injection was observed earlier, in the first week. This might be related to the higher reported levels of VEGF

in the adjacent conjunctiva when compared to cornea.[1,8] Although we could achieve significant regression in corneal vascularization, we could not provide complete resolution. A possible reason for this situation could be that VEGF was shown to lead to the regression of newly formed vessels while it did not cause regression in established neovascularization.[14] Besides, corneal neovascularization is a complex process, which involves other cytokines than VEGF such as transforming growth factor and fibroblast growth factor and is still under investigation. On the other hand, the dosing and frequency of bevacizumab should also be evaluated in future studies with control groups and bigger study population to clarify the place of subconjunctival bevacizumab injection in the treatment armamentarium of corneal neovascularization.

The authors have no proprietary or commercial interest in any materials discussed in this article.

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